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# CELLULARLY GENERATED ACTIVE OXYGEN SPECIES AND HeLa CELL PROLIFERATION

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In HeLa cells evidence is provided that active oxygen species such as hydrogen peroxide and superoxide at low levels are important growth regulatory signals. They may constitute a novel regulatory redox system of control superimposed upon the established cell growth signal transduction pathways. Whilst for example hydrogen peroxide can be added exogenously to elicit growth responses in these cells, it is clear that cellularly generated superoxide and hydrogen peroxide are important. Experiments with superoxide dismutase, superoxide dismutase mimics and inhibitors of both superoxide dismutase and xanthine oxidase suggest that superoxide generated intracellularly and superoxide released extracellularly are both relevant to growth control in HeLa cells.

KEY WORDS: Cell proliferation, superoxide, hydrogen peroxide, catalase, superoxide dismutase, xanthine oxidase, HeLa cells



## INTRODUCTION

Whilst various lines of evidence have suggested that reactions involving free radicals are of significance in at least some carcinogenic mechanisms their particular role in the various processes of tumour 'initiation' and 'promotion' require elucidation. Much evidence comes from observations that agents which scavenge free radicals, or interfere with free radicals mediated events, inhibit the neoplastic processes at the cellular and molecular levels[1]. A notable phenotypic characteristic of tumour cells is reduced levels of certain antioxidant enzymes[1,2]. For example when compared with their appropriate normal counterparts tumour cells are low in manganese superoxide dismutase activity, usually low in copper-zinc superoxide dismutase activity and almost always low in catalase activity. Glutathione peroxidase and glutathione reductase levels however are highly variable. Such observations suggest that tumour cells may be particularly able to accommodate (or accumulate) higher than normal levels of superoxide or hydrogen peroxide. Indeed recent experiments in which increased levels of manganese superoxide dismutase were induced by gene transfer suppressed the malignant phenotype of human melanoma cells[3]. In this context early experiments of ours indicated low concentrations of superoxide or hydroperoxide to be effective in stimulating the growth of oncogene transformed hamster and rat fibroblasts[4,5], which had lower levels of superoxide dismutase activity than their non-transformed counterparts.

It is now clear that superoxide and hydrogen peroxide can stimulate growth and growth responses in a variety of mammalian cell types when added exogenously to the culture medium. Besides hamster and mouse fibroblasts, these include mouse epidermal cells[6], Balb/3T3 cells[7] and human primary fibroblasts[8]. In Balb/3T3

cells[7] and mouse epidermal cells[6], these particular active oxygen species stimulate expression of early growth regulated genes such as the proto-oncogene c-*fos*. These observations have led to suggestions that superoxide and/or hydrogen peroxide might function as mitogenic stimuli through biochemical processes common to natural growth factors.

Whilst a possible source of active oxygen species in *vivo* may be phagocytic cells, there have been recent reports of low levels of superoxide release from a variety of non-inflammatory cells such as human fibroblasts[8,9], endothelial cells[10] and hamster fibroblasts[11]. Hydrogen peroxide release has also been reported from growth stimulated Balb/3T3 cells[7], from pancreactic islet cells[12] as well as from a range of human tumour cells[13].

In a preceding paper[14] we have explored the use of a tetrazolium salt, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to detect superoxide both within HeLa cells and released extracellularly. Resulting from the use of inhibitors of superoxide dismutase and low molecular weight mimics of superoxide dismutase it is suggested that up to 30% of MTT reduced within HeLa cells is due to intracellular superoxide. Whilst a possible source is mitochondria[15] another possibility in HeLa cells, suggested from results with oxypurinol and allopurinol, is xanthine oxidase. About 10% of the total MTT reduction is detected extracellularly in cultures of HeLa cells and at least 80% of this appears to be due to extracellular superoxide although the nature of the source remains to be clarified[14].

Whilst exogenously added superoxide and hydrogen peroxide can stimulate growth responses, a key question is to what extent do cellularly generated active oxygen species contribute to cell proliferation control, particularly of tumour cells. Use of superoxide dismutase, superoxide dismutase mimics and inhibitors, as well as inhibitors of xanthine oxidase, suggest that cellularly generated superoxide and/or hydrogen peroxide have important roles as growth signals in HeLa cells.

## EXPERIMENTAL PROCEDURES

HeLa cells (a gift from Dr M. Stewart, Department of Clinical Biochemistry, Glasgow Royal Infirmary) were grown as monolayer cultures at 37°C in Eagles minimal essential medium (MEM) supplemented with 10% (v/v) calf serum (Gibco BRL, Paisley). Proliferation was assessed as previously described[4,5]. Triplicate monolayer cultures ($0.5 \times 10^6$ cells per 3.5 cm petri dish) were established by growth for 18 hr at 37°C. Thereafter the medium was removed and the cell monolayers washed three times with serum-free medium and then the medium replaced together with the additions indicated in Tables and Figure legends. After 3 days of further growth at 37°C the cells were removed from each dish and yields of live cells assessed using a haemocytometer (live cells were taken as those which did not stain with trypan blue). Results were expressed as yield of cells per dish ± s.d. ($n = 3$).

Copper II (3,5-diisopropylsalicylate)$_2$ was obtained from Aldrich Chemical Co. Diethyldithiocarbamate, nitroblue tetrazolium, oxypurinol, allopurinol, superoxide dismutase (bovine erythrocytes, 5100 U/mg protein) catalase (bovine liver, 3800 U/mg protein) and MTT[3-(4,5-dimethiazol-2-yl)-2,5-diphenyl tetrazolium bromide] were from Sigma Chemical Co.

## RESULTS

Figure 1 shows that as anticipated HeLa cell proliferation can be stimulated by the exogenous addition of low levels of active oxygen species such as $H_2O_2$. An important question however is whether *endogenously* generated active oxygen species could have a similar positive effect on cell proliferation.

In a previous study we used MTT, a tetrazolium salt which permeates cells, to detect intracellular superoxide generation by virtue of its reduction intracellularly to a blue formazan[14]. Although superoxide will act as a reductant of MTT other intracellular reductants may also contribute to the overall levels of MTT reduction. For example work with rat liver suspensions has demonstrated coupling *in vitro* between MTT and two points on the mitochondrial respiratory chain, one being sensitive to antimycin A[16]. Thus it was perhaps not surprising that we observed previously that exposure of HeLa cells to MTT significantly depressed their growth[5]. On the other hand recent studies of ours suggested that at least 30% of the MTT-reduction within HeLa cells may be due to superoxide generated from mitochondria, or from xanthine oxidase[14]. *In vivo* inhibitors of cytosolic Cu,Zn-superoxide dismutase (Cu,Zn-SOD) such as the copper chelator, diethyldithiocarbamate (DDC)[17] were able to increase the rate of MTT reduction within HeLa cells[14] and thus suggested a possible means of increasing intracellular levels of superoxide *in vivo*. Previous experiments showed DDC to inhibit Cu,Zn-SOD activity of HeLa cells *in vivo*[14], effects being observable after 1–2 hr exposure. As evident from Figure 2, DDC addition to growing cultures of HeLa cells, at least low levels, caused a growth stimulation similar to that encountered upon addition

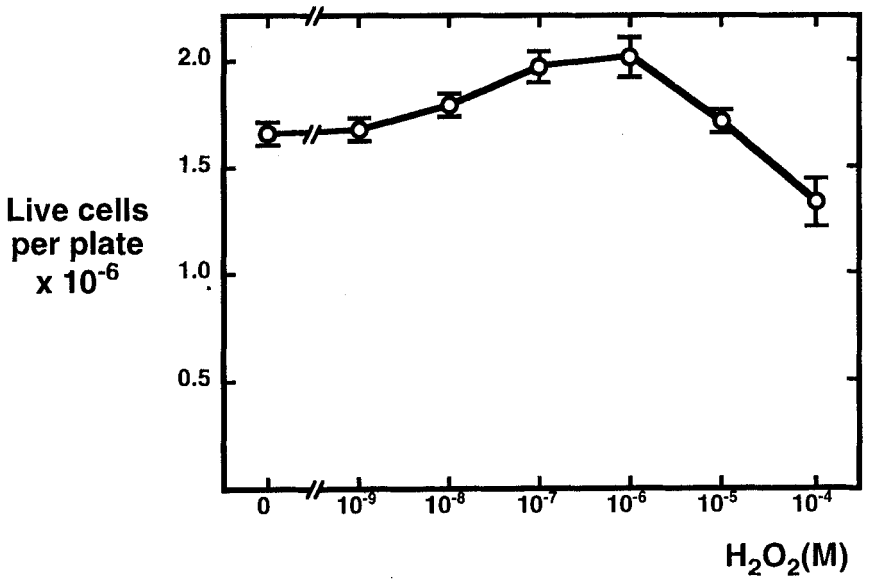


FIGURE 1 The effect of hydrogen peroxide on the growth of HeLa cells. Triplicate monolayer cultures of HeLa cells were established as described in Experimental Procedures. The medium was then removed and replaced with fresh medium containing hydrogen peroxide at various concentrations. After 3 days of further growth at 37°C live cells were assessed and the data presented as described in EXPERIMENTAL PROCEDURES.

of exogenous $H_2O_2$. In contrast if DDC addition was carried out in the presence of MTT no growth stimulatory effects were observed (Figure 2).

In our previous studies we also were able to diminish cellular MTT-reduction by inclusion in the growth medium, of the lipophilic superoxide dismutase mimic, CuDIPS [copper II(3,5-diisopropyl salicylate)$_2$][18]. Figure 3 shows that CuDIPS also markedly reduces the proliferation of HeLa cells. Growth reduction was also observed if *exogenous* Cu,Zn-SOD (from bovine erythrocytes) was added to the growth medium (Figure 4). Whilst this enzyme may remain outside the cultured cells there are a growing number of reports to indicate its endocytosis by various cell types[19-22]. Catalase addition to the growth medium was even more inhibitory (see also Figure 4). The inhibitory effects of exogenous Cu,Zn-SOD and catalase were not additive and were almost eliminated by prior heat treatment of the enzymes (25 min 100°C) (Table 1). Another class of inhibitors of HeLa cell growth were the xanthine oxidase inhibitors[23] oxypurinol and allopurinol (Table 2 and Figure 5). The inhibitory effects of oxypurinol and CuDIPS were not additive (Table 2).

TABLE 1
Effect of exogenous superoxide dismutase and catalase on HeLa cell growth

| Additions | Live cells/plate after 3 days × $10^{-6}$ ± s.d. |
|---|---|
| none | 1.48 ± 0.05 |
| superoxide dismutase (100 μg/ml) | 1.06 ± 0.02 |
| catalase (100 μg/ml) | 0.78 ± 0.05 |
| * heat-treated superoxide dismutase (100 μg/ml) | 1.40 ± 0.05 |
| * heat-treated catalase (100 μg/ml) | 1.35 ± 0.02 |
| superoxide dismutase (100 μg/ml) plus catalase (100 μg/ml) | 0.85 ± 0.06 |

Triplicate monolayer cultures of HeLa cells (0.5 × $10^6$ cells per 3.5 cm petri plate) were established as described in EXPERIMENTAL PROCEDURES. The medium was then replaced together with the additions indicated and incubation continued at 37°C for a further 3 days. The live cells in each plate were counted and the results presented are the means of triplicate experiments ± s.d. (n = 3). [* heat treatment 100°C, 25 min].

TABLE 2
Effect of xanthine oxidase inhibitors on HeLa cell growth

| Expt | Additions | Live cells/plate after 3 days × $10^{-6}$ ± s.d. |
|---|---|---|
| 1 | none | 1.55 ± 0.05 |
| | oxypurinol (10 μM) | 1.34 ± 0.06 |
| | allopurinol (10 μM) | 1.01 ± 0.07 |
| 2 | 0.1% ethanol | 1.38 ± 0.02 |
| | 0.1% ethanol + CuDIPS (10 μM) | 1.10 ± 0.03 |
| | 0.1% ethanol + CuDIPS (10 μM) + oxypurinol (10 μM) | 1.17 ± 0.03 |

Triplicate monolayer cultures of HeLa cells (0.5 × $10^6$ cells per 3.5 cm petri plate) were established as described in Experimental Procedures. The medium was then replaced together with the additions indicated and incubation continued at 37°C for a further 3 days. The live cells in each plate were counted and the results presented are the means of triplicate experiments ± s.d. (n = 3) [CuDIPS, CuII(3,5-diisopropylsalicylate)$_2$].

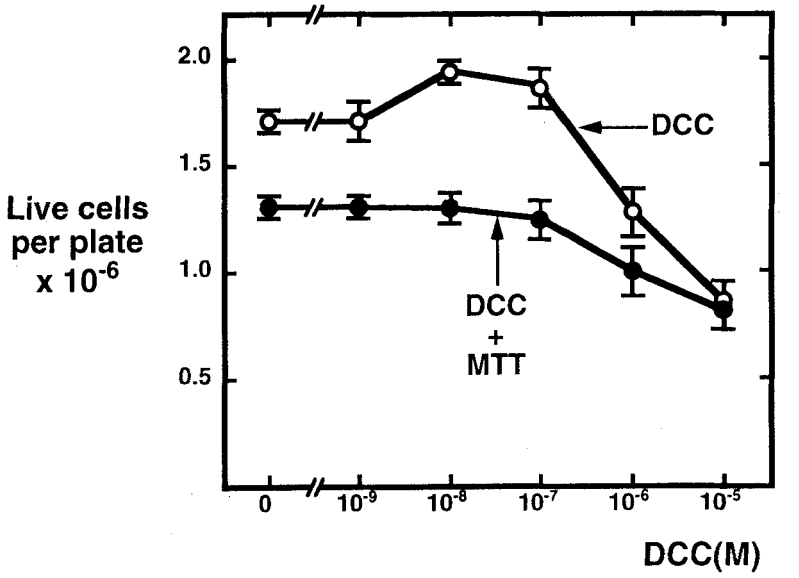


FIGURE 2 The effect of diethyldithiocarbamate and the tetrazolium salt, MTT, on the growth of HeLa cells. Cell cultures were established as in Figure 1 and after removal of the medium it was replaced with fresh medium containing varying levels of diethyldithiocarbamate (DDC) with (●) or without 125 μg/ml MTT (○). Assessment of cell growth was as in Figure 1.

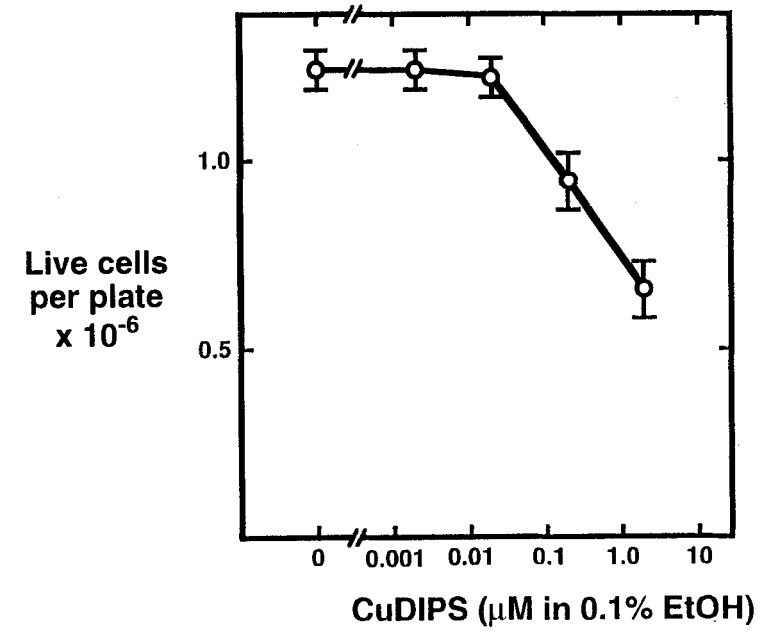


FIGURE 3 The effect of copperII (3,5-diisopropyl salicylate)$_2$ on the growth of HeLa cells. Cell cultures were established as in Figure 1 and after removal of the medium it was replaced with fresh medium containing varying levels of copperII (3,5-diisopropylsalicylate)$_2$ (CuDIPS). Assessment of cell growth was as in Figure 1.

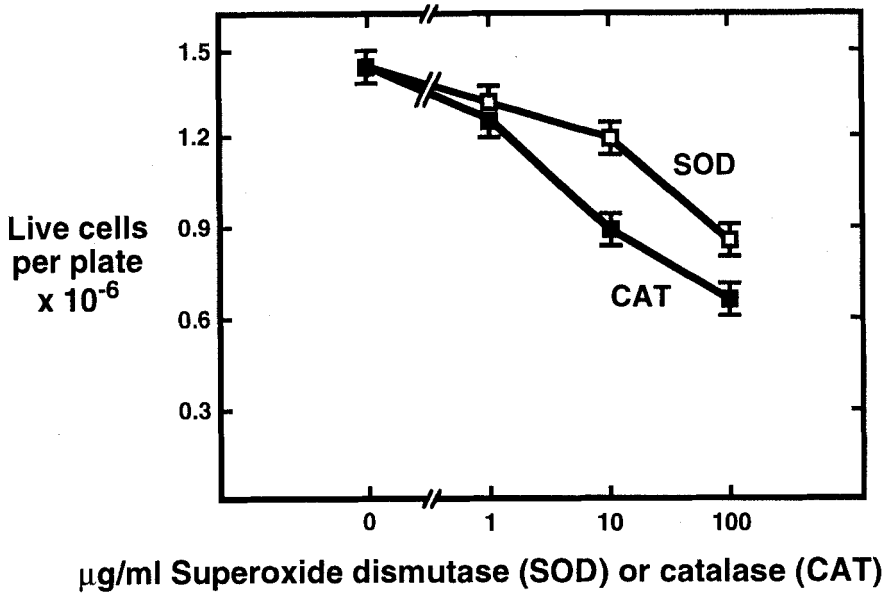


FIGURE 4 The effects of Cu, Zn-superoxide dismutase and catalase on the growth of HeLa cells. Cell cultures were established as in Figure 1 and after removal of the growth medium, fresh medium was added containing various levels of Cu,Zn-superoxide dismutase (SOD) or catalase (CAT). Assessment of cell growth was as described in Figure 1.

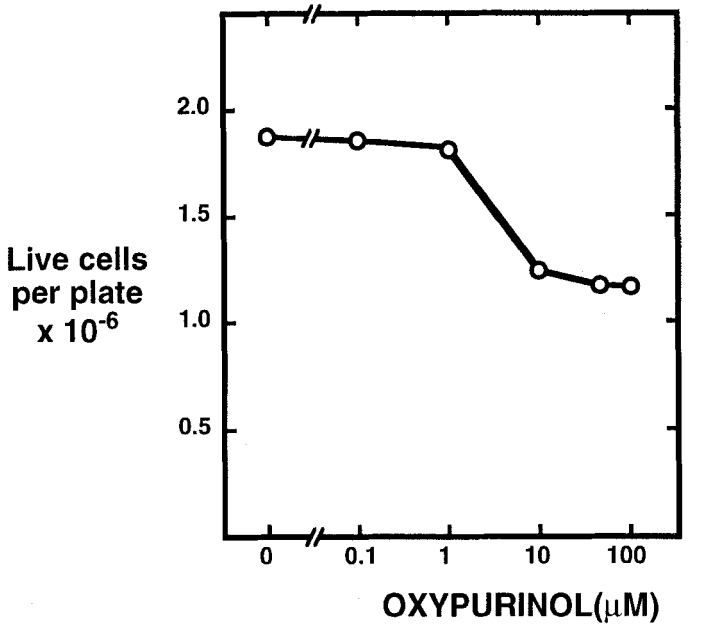


FIGURE 5 The effect of oxypurinol on HeLa cell growth. Cell cultures were established as in Figure 1 and after removal of the growth medium, fresh medium was added containing various levels of oxypurinol. Assessment of cell growth was as described in Figure 1.

## DISCUSSION

The observation that MTT significantly inhibits growth of HeLa cells may be due *both* to its ability to interact directly with components of the mitochondrial respiratory chain and its ability to interact with intracellularly generated superoxide anions. The Cu,Zn-SOD inhibitor DDC was used to determine whether an agent which might be expected to cause an accumulation of intracellular superoxide might in turn affect cell growth. At low levels, DDC caused a modest growth stimulation although a down-regulation of growth was observed at higher concentrations of DDC (Figure 2). However if MTT is included in the growth medium along with the DDC, the stimulatory effects of low levels of DDC are not observed. It is possible that MTT simply interacts with any additional intracellular superoxide made available by virture of the inhibition of the cytosolic Cu,Zn-SOD. With regard to the down-regulatory effects of DDC at higher concentrations, DDC itself may scavenge reactive oxygen species, or by virtue of its inhibition of superoxide dismutase, depress cellular levels of hydrogen peroxide which as argued below is also important for cell proliferation. Other means of increasing intracellular superoxide can also lead to growth stimulatory effects. For example in a previous report we observed that paraquat at low concentrations could stimulate the growth of oncogene-transformed rat fibroblasts (RFAGT1)[5]. Paraquat within cells is reported to undergo repeated cycles of oxidation and reduction[24] to serve as a continuous additional intracellular source of superoxide anions.

With regard to intracellularly produced superoxide anions, the action of cellular superoxide dismutases would yield $H_2O_2$ which is also growth promoting, at least when added exogenously to HeLa cells (eg. Figure 1). Unlike superoxide, $H_2O_2$ can freely permeate cell membranes [24]. Whilst both exogenous superoxide and $H_2O_2$ can promote general cell proliferative responses, there are nevertheless some differences which may be important. For example unlike $H_2O_2$, superoxide will very rapidly increase the intracellular pH and $Ca^{2+}$ levels in human amnion cells.[25] These rapid responses elicited by superoxide are nevertheless inhibited by drugs that block the activity of anion channels suggesting that such channels are an essential means whereby exogenous superoxide may gain entry within cells[25].

In order to clarify further the significance of *endogenous* superoxide as distinct from *exogenous* superoxide as a growth promoter in HeLa cells, use was made of CuDIPS, the lipophilic low molecular weight mimic of superoxide dismutase[18]. This notably reduced intracellular superoxide[14] levels as well as HeLa cell proliferation supporting the view that endogenously generated superoxide might have an important growth regulatory role. Since we had previously suggested that xanthine oxidase may be a source of endogenous superoxide in HeLa cells, the growth inhibitory effects of oxypurinol and allopurinol on HeLa cells were therefore not unexpected. Again these observations support the view that intracellularly generated superoxide (or derived active oxygen species) are critical for the growth regulation of HeLa cells. On a more general note such observations may have a wider significance for tumour cells in general which are reported to have reduced levels of both superoxide dismutase and catalase[1,26] thus potentially permitting a greater intracellular accumulation of growth stimulatory superoxide and hydrogen peroxide. Whilst we find that the major proportion (80%) of cellular MTT reduction attributable to superoxide appears to be due to *intracellular* superoxide[14], an important question is whether the intracellular superoxide is more important for growth regulation than the superoxide released extracellularly. Previous experiments with

rat and hamster fibroblasts and using nitroblue tetrazolium (NBT), another tetrazolium salt that can be reduced by superoxide, but which does not penetrate cells, indicated that *extracellular* superoxide is of considerable importance for the proliferation of these cells[11]. Exposure of HeLa cells to NBT also reduced their proliferation (Figure 6) thus supporting the importance of *extracellular* superoxide for HeLa cell proliferation.

Another critical observation is that the addition of exogenous superoxide dismutase or catalase to cultures of HeLa cells were *both* growth inhibitory. This was previously observed when these enzymes were added to cultures of transformed hamster (BHK-21/PyY) and rat (RFAGT1) fibroblasts[11]. Such experiments suggest that *both* superoxide and $H_2O_2$ are important growth signals for modulation of cell proliferation. Moreover the observation that the inhibitory effects of exogenous superoxide dismutase and catalase are not additive indicates that removal of either superoxide or hydrogen peroxide has similar 'down-regulatory' effects on cell proliferation and that *both* signals are important for growth. Whilst the particular catalase preparation used in this study contained a low level of contaminating superoxide dismutase-like activity (see also ref 27), the down-regulatory effects of catalase addition on HeLa cell growth that we observed were *always* greater than those of exogenously added superoxide dismutase, suggesting that such a contaminant activity is not a simple explanation of the observed catalase effects. Moreover as Table 1 shows the effects of superoxide dismutase addition *together* with catalase are not additive. Indeed in the case of HeLa cells the combination of enzymes is slightly less effective than catalase on its own. In addition whereas the superoxide dismutase-like activity present in the bovine liver catalase preparation is abolished

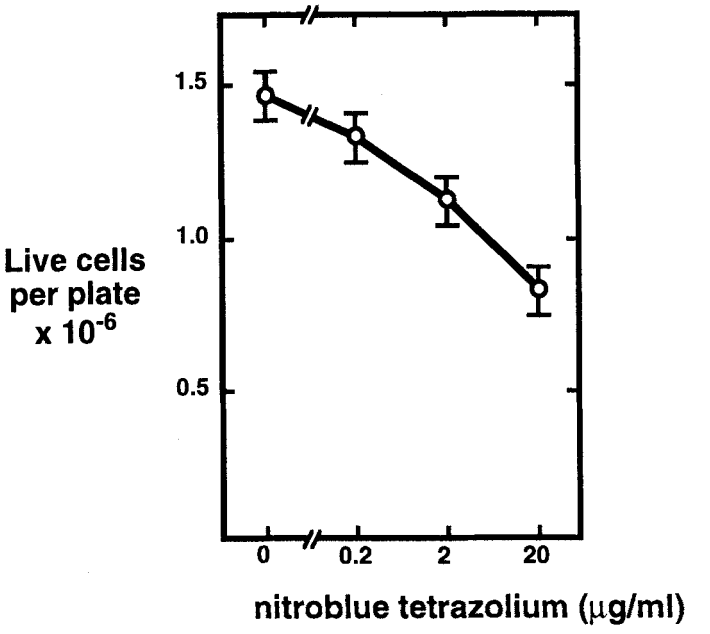


FIGURE 6 The effect of nitroblue tetrazolium in HeLa cell growth. Cell cultures were established as in Figure 1. After removal of the growth medium, fresh medium was added containing various levels of nitroblue tetrazolium (NBT). Assessment of cell growth was as described in Figure 1.

by heat treatment at 100°C for 2 min (see also ref 27), the down-regulatory effects of catalase on HeLa cell growth are not completely eradicated after 25 min at 100°C (see Table 1).

At first sight these experiments would appear to support the view that *extracellular* superoxide and hydrogen peroxide are of prime importance. However as many cell types are known to take up superoxide dismutase by endocytosis[19-22], such experiments do not rule out a contribution to growth control from *intracellular* as well as *extracellular* superoxide. The observation that quite high levels of superoxide dismutase or catalase are required for growth inhibition may be a reflection of some involvement of endocytosis in eliciting the growth inhibitory effects of these exogenously added antioxidant enzymes.

In summary these experiments lend further support for the view that in HeLa cells, as well as in BHK-21/PyY and RFAGT1 cells[11,28], both superoxide and hydrogen peroxide are important growth regulatory signals. Moreover it appears that both extracellular and intracellular superoxide may be important in this context. On a mechanistic basis our views of cell communication are presently dominated by a simple paradigm that signalling is accomplished by molecules that bind non-covalently to specific receptors through complementarity of shape. It is possible that there is a quite different variety of cellular signalling in which the 'signal molecule' interacts with a target molecule covalently on the basis of its redox potential. Whilst studies have shown that the activation of protein kinase C for example is an important component of the pathway leading to the activation of the pathway leading to the induction of the early growth response gene c-*fos* in certain cells[29] it is possible that $H_2O_2$ could directly modulate protein kinase C. Activation of protein kinase C and translocation of protein kinase C is an early response to oxidant growth stimulation *in vivo*[28] and protein kinase C activation has been achieved *in vitro* by selective oxidative modification of the regulatory domain[31]. The activity of other cellular protein kinases can be modified by redox changes[32] and the same seems to be the case of certain transcription factors such as Fos/Jun and NF-kappa B and Ets[33-37]. Such observations suggest the possibility of a novel redox growth regulatory paradigm superimposed upon the established cellular signal transduction pathways. It may be that adjustment of the redox states of proteins involved in these pathways is a prerequisite for their optimal functioning. In the case of tumour cells the lower levels of superoxide dismutase or catalase[1,3,5,26], might create conditions for the intracellular accumulation of superoxide and hydrogen peroxide which in turn might permit the setting of a cellular 'redox state' that facilitates the continuous 'up-regulation' of the signal transduction proteins that promote deregulated tumour cell growth.

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